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## Antigenic Variation of Core, NS3, and NS5 Proteins among Genotypes of Hepatitis C Virus

J. A. NEVILLE,<sup>1</sup> L. E. PRESCOTT,<sup>1</sup> V. BHATTACHERJEE,<sup>1</sup> N. ADAMS,<sup>1</sup> I. PIKE,<sup>2</sup> B. RODGERS,<sup>2</sup>  
A. EL-ZAYADI,<sup>3</sup> S. HAMID,<sup>4</sup> G. M. DUSHEIKO,<sup>5</sup> A. A. SAEED,<sup>6</sup>  
G. H. HAYDON,<sup>7</sup> AND P. SIMMONDS<sup>1\*</sup>

*Department of Medical Microbiology, University of Edinburgh,<sup>1</sup> and Department of Medicine, University of Edinburgh, Royal Infirmary of Edinburgh,<sup>7</sup> Edinburgh, Murex Biotech Ltd., Dartford, Kent,<sup>2</sup> and Department of Medicine, Royal Free Hospital, London,<sup>5</sup> United Kingdom; Cairo Liver Centre, Dokki, Giza, Egypt<sup>3</sup>; Department of Medicine, Aga Khan University, Karachi, Pakistan<sup>4</sup>; and Department of Pathology, Riyadh Armed Forces Hospital, Riyadh, Saudi Arabia<sup>6</sup>*

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**Assays that detect antibody to hepatitis C virus (HCV) are used to screen blood donors and patients with hepatitis. Current enzyme-linked immunosorbent assay (ELISA)-based methods are invariably based upon antigens from expressed recombinant proteins or oligopeptides from HCV type 1. Some HCV antigens used in screening assays are coded by regions of the HCV genome that show extensive variability; therefore, HCV type 1-based assays may be less effective for the detection of antibody elicited by infection with other genotypes. In this study, we have measured antibody reactivity of sera from 110 hepatitis C patients infected with type 1b, 3a, or 4a to genotype-specific and cross-reactive epitopes present in recombinant proteins from HCV genotypes 1b (core, NS3, and NS5), 3a (NS3, NS5), and 4a (core, NS3), corresponding to those used in current third-generation screening ELISAs. By comparing the serological reactivities of sera to type-homologous and type-heterologous antigens, we detected a significant type-specific component to the reactivity to NS3 (61 to 77% of the total reactivity) and NS5 (60% of the total reactivity). Furthermore, despite the similarities in the amino acid sequences of the core antigens of type 1b and type 4a, we also found significantly greater reactivity to type-homologous antigens, with approximately 25% of reactivity being type specific. These findings are consistent with previous findings of fivefold weaker reactivity of sera from HCV type 2- and HCV type 3-infected blood donors in the currently used third-generation ELISAs and suggest that these assays are suboptimal for screening populations in which the predominant genotype is not type 1.**

Detection of antibody to hepatitis C virus (HCV) has become the principal method for the diagnosis of HCV infection in individuals with chronic hepatitis and for the screening of blood donors. Although the original assay based upon the c100-3 recombinant proteins derived from NS4 showed non-specificity and insensitivity, the more recently developed assays that use recombinant proteins from the core and NS3 regions of the HCV genome (second-generation assays) and the NS5 region of the HCV genome (third-generation assays) have proved to be more effective for the screening of blood donors. Their use has led to a substantial reduction in the incidence of posttransfusion hepatitis. In prospective studies, the incidence of HCV transmission among recipients of blood screened by first-generation assays was 1.5% in Spain (15), 3.7% in Japan (27), and 11% in Taiwan (6). Screening by second-generation assays reduced or would have reduced the incidence to 0.9% in Japan (27), 1% in Spain (15), 2% in Greece (20), and 2.5% in Taiwan (6).

One reason for the failure to detect antibody to HCV in donated blood specimens that transmit HCV infection is that the blood was collected from an individual with acute infection before seroconversion for antibody. This so-called “window” period is long for HCV compared with other viruses for which blood from donors is screened, such as human immunodeficiency virus, with means of 88 to 66 days in second- and third-

generation assays, respectively (9). By measuring the incidence of HCV infection in blood donors, it has been estimated that the current residual risk of HCV transmission through collection of “window” samples is 1 per 100,000 donations in the United States (24). This frequency of infection is similar to the residual risk of infection from blood screened by third-generation assays from French blood donors (10).

In addition, samples false negative for antibody have been reported for a small proportion of immunocompetent individuals persistently infected with HCV (2, 17). Indeed, even in anti-HCV-positive individuals, there is considerable variability in the frequency of reactivity to the individual HCV proteins used in supplementary assays, such as the Ortho 3rd generation recombinant immunoblot assay (RIBA-3), and in the titer of antibody to HCV among persistently infected individuals. Among 90 anti-HCV-positive blood donors, antibody reactivity in the Ortho third-generation screening assay varied over a range of 5,000-fold, with some serum samples having antibody levels only just above the cutoff for the assay (12).

HCV can be classified into at least six major genotypes, whose nucleotide and inferred amino acid sequences over the whole genome differ by approximately 30%. This degree of amino acid sequence variability is similar to that observed between variants of other RNA viruses (e.g., dengue virus types 1 to 4), in which significant antigenic differences have been documented and which form the basis of their classification into serotypes. We have previously measured the serological reactivities of individuals infected with different HCV genotypes to antigens used in two third-generation anti-HCV assays (Ortho 3rd generation anti-HCV EIA; Murex VK48).

\* Corresponding author. Mailing address: Department of Medical Microbiology, University of Edinburgh, Teriot Place, Edinburgh, United Kingdom. Phone: 44 131 650 3138. Fax: 44 131 650 6531. E-mail: Peter.Simmonds@ed.ac.uk.

Samples from HCV type 1-infected individuals showed an approximately five times greater reactivity than those infected with HCV type 2 or 3, and the reactivity was independent of other factors that may have influenced the antibody response, such as the degree of viremia, donor age, and severity of hepatitis as assessed by alanine aminotransferase measurements.

In the current study we have addressed two of the main shortcomings of the first study. First, we have measured serological reactivities to the individual component antigens used in a third-generation assay (core, NS3, and NS5). These measurements extend previous investigations of type-specific and cross-reactive serological reactivity to the NS4 antigen used in the first-generation screening assays and in second- and third-generation confirmatory assays (1, 5, 14, 21, 26, 28). Second, we have carried out titrations with antigens derived from HCV type 1 as well as corresponding proteins from other HCV genotypes (types 3a and 4a). This allows levels of antibodies to both type-homologous and type-heterologous antigens to be measured, allowing for a more rigorous assessment of the relative levels of type-specific and type-common reactivity to each.

#### MATERIALS AND METHODS

**Samples.** Samples LJ516, EG21, and ED43 were obtained from individuals infected with genotype 3a (LJ516) or 4a (EG21 and ED43 [3, 25]). Sequences amplified from the core, NS3, and NS5 regions of types 3a and 4a were used for the synthesis of antigens for antibody screening. Recombinant antigens from the core (amino acid positions 1 to 140), NS3 (amino acid positions 1360 to 1454), and NS5 (amino acid positions 2234 to 2318) (numbered according to Choo et al. [7]) regions of HCV-UK (type 1b) were derived from an existing, commercially available anti-HCV assay (VK48).

Serum samples were obtained from 110 anti-HCV-positive individuals with chronic hepatitis C attending hospital liver clinics in Edinburgh and London, United Kingdom; Karachi, Pakistan; and Cairo, Egypt. Among the samples, we selected 33 infected with genotype 1b, 34 infected with genotype 3a, and 43 infected with genotype 4a. Genotypes were identified by restriction fragment length polymorphism analysis of the 5' noncoding region as described previously (11).

**Development of enzyme-linked immunosorbent assays (ELISAs) based upon type 3a and 4a antigens.** RNAs from samples LJ516 (type 3a), EG21 (type 4a), and ED43 (type 4a) were extracted as described previously (16). Reverse transcription was carried out with avian myeloblastosis virus reverse transcriptase and RNasin (both from Promega, Southampton, United Kingdom), in each case by using the external antisense primer, and incubation at 42°C for 30 min as described previously (4). The sequences were amplified by using the following heat cycle: 94°C for 18 s, 45°C for 21 s, and 72°C for 90 s for 30 cycles, followed by 6 min at 72°C with the primers listed in Table 1. The core region of type 4a was amplified with primers 954 and 410, followed by amplification with primers D236 and 597. The NS3 region was amplified with primers 751 and 753, followed by amplification with primers 594 and 593 (type 3a) or 750 and 007 and then primers 594 and 593 (type 4a). For the type 4a NS3 region, a third PCR was carried out with the product of the second PCR by using the same inner primers. The NS5 region of genotype 3a was amplified by using primers 991 and 993, followed by amplification with primers 3155 and 3156. The amplified sequences were cloned and sequenced as described previously (23).

**Expression of recombinant proteins.** The sequences of the core, NS3, and NS5 regions from the different HCV genotypes indicated above were subcloned into *Escherichia coli* or baculovirus expression vectors, as appropriate, by standard molecular biology techniques. The NS3 and NS5 sequences of each genotype were cloned into the *E. coli* vector pTrcHis (Invitrogen BV, Leek, The Netherlands) and were expressed in TOP10 cells by standard isopropyl- $\beta$ -D-thiogalactopyranoside induction. Core sequences were cloned into the baculovirus expression vector pBlueBacHis2 (Invitrogen), and recombinant virus was derived by cotransfection into insect cells along with linear *Antographa californica* nuclear polyhedrosis virus DNA. Recombinant antigens were produced in Sf.9 insect cells in an LH Series 210 bioreactor by infection at multiplicity of infection of 2 for 48 h.

Both the *E. coli* and baculovirus vector expression vectors place a tract of six histidine residues at the amino terminus of each recombinant protein. Recombinant proteins were therefore subsequently prepared by metal chelate chromatography on Probond nickel affinity resin (Invitrogen) to approximately 90% purity.

**ELISA for detection of antibody to core, NS3, and NS5 regions.** To optimize plate coating, purified antigens were titrated to levels giving acceptable specificity and sensitivity and approximately equivalent amounts of protein per well (25 to

TABLE 1. Primers used for amplification of core, NS3, and NS5 regions

Primer no.	Sequence <sup>a</sup>
954.....	ACT GCC TGA TAG GGT GCT TGC GAG
410.....	GAG CGG AAT GTA CCC CAT GAG GTC GGC
D236.....	GGT AGA CCG <u>GAT</u> CCC ATG AGC ACG AAT CCT AAA
597.....	GAA AGA <u>AGC</u> TTA GAC GAG CGG AAT GTA CCC
751.....	TTY CGG GCN GCY GTG TGC ACC
753.....	GCG TCA TAG CAC TCA CAG AGG AC
594.....	ACC RCT <u>GCA</u> GAY CCY AAC ATN GAG GAR GT
593.....	CAC <u>AAG</u> CTT ARC AGT AGT CKA TSA CNG ART C
750.....	TGG TYA CNN GNN AYG CYG ATG TCA TYC C
007.....	ACC TCG AGT ATC CCA CTG ATG AAG TTC CAC AT
991.....	TGG CNA GCT CNT CNG CNA GCC A
993.....	GGC TCC CCC TCA AGG GRG GCA TRG A
3155.....	<u>GGA</u> TCC AAY ATN ACY CGY GTR GAG TCW SA
3156.....	<u>AAG</u> CTT GTG GTG GYA AGG CAC AYC C

<sup>a</sup> International Union of Pure and Applied Chemistry ambiguity codes were used: Y, C/T; R, A/G; N, G/C/A/T; K, G/T; S, C/G; W, A/T. Cleavage sites for restriction enzymes are underlined: CTGCAG, *Pst*I; AAGCTT, *Hind*III; GGA TCC, *Bam*HI.

50 ng). Binding of antigen was assessed both by functional enzyme immunoassay (EIA) with positive and negative sera and by probing for the histidine tag present on each protein by using horseradish peroxidase chemically modified to bind nickel ions. All antigens were coated overnight in 50 mM Tris (pH 8.5) containing 0.02% sodium dodecyl sulfate. The plates were then blocked by the addition of 2% degraded gelatin and were finally dried to ensure stability.

For each antigen assay, each serum sample was diluted 1:10, 1:40, 1:160, and 1:640 in anti-HCV-negative sera. Aliquots of 20  $\mu$ l from each titration were added to 180  $\mu$ l of sample diluent, mixed, and then added to a well of the ELISA plate. A solution of 1:50 *E. coli* blocker was added to the diluent for all assays for NS3 genotype 3a antigen. The plates were then incubated at 37°C for 1 h, followed by five washes in glycerate-borate buffer. Bound antibody was detected by the addition of 100  $\mu$ l of horseradish peroxidase-labelled antibody to human immunoglobulin G conjugate and by incubation for 30 min at 37°C. After washing, 100  $\mu$ l of 3,3',5,5'-tetramethylbenzidine substrate was added, and the mixture was incubated for 30 min at 37°C. The color development reaction was stopped by the addition of 50  $\mu$ l of 2 M H<sub>2</sub>SO<sub>4</sub>, and optical densities (ODs) were measured at 470 nm. All assay plates included a dilution series of the reference genotype 1a sample (dilutions of 1:2 to 1:2,560) for calculation of antibody levels. The NS3 genotype 3a recombinant antigens were expressed in *E. coli* and required the addition of 1:50 *E. coli* blocking solution in the sample diluent for all assays.

**Quantification of antibody levels.** Antibody reactivity to each recombinant protein in each of the test serum samples was determined by titration and reference to a standard positive control as described previously (12). This method is based upon the observation of a linear relationship between OD (over the range of values of 0.02 to 1.0) and concentration of antibody in both ELISAs based upon single antigens, as in the current study and in the currently used third-generation screening assays from Ortho and Murex (12). Because of this relationship, the antibody reactivity of a test sample can be expressed relative to that of a reference control by the following formula: test antibody level = (test OD  $\times$  reference concentration)/(reference OD  $\times$  test concentration). Antibody levels were obtained for each sample in each antigen assay in this way.

**Estimation of relative proportions of type-specific and cross-reactive antibodies.** The ratio of antibody levels measured to type-homologous and type-heterologous antigens indicates the relative proportions of type-specific and cross-reactive reactivities. This calculation is complicated by the possible existence of differences in the antigenicity or presentation of proteins from different genotypes in the ELISA and differences in antibody levels elicited by infection with different genotypes.

The proportion of antibody reactivity that is cross-reactive between genotypes is measured by reciprocal assays of reactivity between sera and antigens from two genotypes, genotypes A and B. In the following relations,  $E_{BA}$  represents the antibody reactivity of a serum sample infected with genotype B with antigen of genotype A,  $A_{AB}$  is the overall antigenicity of the genotype A antigen relative to that of the genotype B antigen, and  $T_c$  and  $T_s$  are the proportions of the antibody response that are type common and type specific, respectively, such that  $T_c + T_s = 1$ .

$$\frac{E_{BB}}{E_{BA}} = \frac{E_{BB}}{E_{BB} \times A_{AB} \times T_c} = \frac{1}{A_{AB} \times T_c}$$

$$\frac{E_{AB}}{E_{AA}} = \frac{E_{AA} \times (1/A_{AB}) \times T_c}{E_{AA}} = \frac{T_c}{A_{AB}}$$

A		1	21	41	61	70	
1a HCV-PT	MSTNPKPKQKK	NKRNTNRRPQ	DVKFPGGGQI	VGGVYLLPRR	GPRLGVRATR	KTSERSQPRG	RRQPIPKARR
1a HC-J1	...I...R.	T.....	.....	.....	.....	.....	.....V..
1b HCV-J	.....R.	T.....	.....	.....	.....	.....	.....
1b BK	.....R.	T.....	.....	.....	.....P.	.....	.....
HCVUPCP	.....R.	T.....	.....	.....	.....	.....	.....Q
1c HC-J9	.....R.	T.....	.....	.....	.....V.....	.....	.....
2a HC-J6	.....R.	T.....	.....	.....	.....	.....	.....D..
DX503P2A	.....R.	T.....	.....	.....	.....T..	.....	.....Q.....D..
2b HC-J8	.....R.	T.....	.....	.....	.....	.....	.....D..
2c BEBE1	.....R.	T.....	.....	.....	.....A.	.....	.....D..
3a NZL1	...L...R.	T...I...	.....	.....V.....	.....	.....	.....
3a HPCHK6	...L...R.	T...I...	.....V.	YV...V.....	.....	.....	...K.....
3b Tr	...L...KRQ	T...L...K	N...A...	...E...V...	...Q...EV.	.....	...T...P
10a JK049	...L...RI	T...I...	.....	...V...K...	...V.....	.....S	...R...
4a ED43	.....R.	T.....M	.....	.....	.....	.....	.....
DX387C4A	.....R.	T.....M	.....	.....	.....	.....	.....
11a JK046	.....RQ	T.....	.....	.....	.....	.....	.....
DX461C5P	.....R.	T.....	.....	.....D.....	.....	.....	.....Q
		71	91	111	131	140	
1a HCV-PT	PEGRTWAQPG	YPWPPLYGNEG	CGWAGWLLSP	RGSRPSWGPT	DPRRRSRNLG	KVIDTLTCGF	ADLMGYIPLV
1a HC-J1	.....	.....	.....	.....	.....	.....	.....
1b HCV-J	.....	.....	M.....	.....	.....	.....	.....
1b BK	.....	.....	L.....	.....	.....	.....	.....
DXUPCP	...A.....	.....M.....	.....	.....	.....	.....	.....
1c HC-J9	...S.....	.....	.....	.....S	.....	.....	.....
2a HC-J6	ST.KS.GK..	.....L.....	.....	.....N	...H...V.	.....	.....V.
DX503	STDKS.GK..	.....L.....	.....	.....	...H...V.	.....	.....
2b HC-J8	ST.KS.GK..	.....	.....	.....T...	...H...R...	...I.....	.....V.
2c BEBE1	ST.KS.GR..	.....R...L.....	.....	.....S	...HK.....	.....	.....V.
3a NZL1	S...S.....	.....	.....	.....N	.....	.....	.....
3a HPCHK6	S...S.....	.....	.....	...N.A.N	.....	.....	.....
3b Tr	R...S.....	.....P.	.....	...N.QN	.....	.....	...I.....
10a JK049	T...S.....	.....	.....	.....	.....	.....	.....
4a ED43	...S.....	.....	.....	.....N	...G.....	.....	.....
DX387	S...S.....	FA.....	.....	.....N	.....	.....L	.....
11a JK046	QT...A.G...	...A.....	.....	.....T...N	.....	.....L	.....VI
DX461	...T...S.G...	...A...L.....	.....	.....N...N	...K.....	.....	.....

FIG. 1. Comparison of the inferred amino acid sequences of antigens expressed from recombinant clones of (A) core (HCVUPCP, type 1b; DX387, type 4a), (B) NS3 (HCVUPCP, type 1b; DX506, type 3a; DX499, type 4a), and (C) NS5 regions (HCVUPCP, type 1b; DX507, type 3a) with representatives of each of the major HCV genotypes and subtypes. Periods indicate identity with the HCV prototype sequence, HCV-PT (7). Numbers refer to amino acid positions on HCV-PT.

Therefore,

$$\frac{E_{BB}/E_{BA}}{E_{AB}/E_{AA}} = \frac{A_{AB}}{A_{AB} \times (T_c)^2} = \frac{1}{(T_c)^2}$$

From this the proportion of type-common reactivity can be derived as follows:

$$T_c = \sqrt{\frac{E_{AB}/E_{AA}}{E_{BB}/E_{BA}}}$$

and

$$A_{AB} = \sqrt{\frac{1}{(E_{AA}/E_{AB}) \times (E_{BA}/E_{BB})}}$$

These relations make the assumption that the relative proportions of type-specific to type-common reactivity in a type A antiserum are the same as those found in a type B antiserum.

**Nucleotide sequence accession numbers.** The sequences obtained in the course of this project have been submitted to GenBank and bear the following

accession numbers: DX387, AFO29298; DX506, AFO29297; DX499, AFO29296; DX507, AFO29299.

## RESULTS

**Variability of core, NS3, and NS5 region sequences.** The amino acid sequences of the recombinant proteins used in the ELISA were deduced from the nucleotide sequences of the corresponding clones and were compared with other published sequences of these regions of HCV (Fig. 1). In the core protein only 9 amino acids differed between genotypes 1b and 4a over the 140-amino-acid length (6% divergence). Substitutions were generally conservative, where only one resulted in a change in the ionic charge. The NS3 sequences exhibited more variability, with the sequence of genotype 1b differing from that of genotype 3a by 15 of 95 amino acids (16% divergence) and from that of genotype 4a by 13 amino acids, while genotypes 3a

B		1360	1370	1380	1390	1400	1410	1420	1430	1440	1450
1a HCV-PT	PNIEEVALST	TGEIPFYGKA	IPLEVIKGR	HLIFCHSKKK	CDELAALKVA	LGINAVAYYR	GLDVSVIPTS	GDVVVVATDA	LMTGYTGDFD	SVIDC	
1a HC-J1	A.....	.....A.....	.....	.....	.....	.....V.....	.....	.....	.....	.....	
1b HCV-J	.....N.....	.....	..I.A.....	.....	.....TG..	..L.....	.....	.....	.....F.....	.....	
1b BK	.....N.....	.....	..I.A.R.....	.....	.....SG..	.....	.....I.....	.....	.....	.....	
HCVUPCP	.....N.....	.....	..I.T.....	.....	.....G.....	.....	.....A.....	.....	.....F.....	.....	
1c HC-J9	S.....	E.....	..NY.....	.....	.....G.....	..V...F..	.....T.....	.....	.....	.....	
2a HC-J6	.....GQ	E.....R.	..SY.....	.....	.....A.RG	M.L.....	.....Q.....	.....	.....F.....	.....	
2b HC-J8	S.....GH	E.....	..AF.....	.....	.....A.RG	M.V.....	.....Q.....	.....	.....	.....	
2c BEBE1	.....GH	E.....	..SA.....	.....	.....VA.RG	M.L.....	.....I...Q.....	.....	.....	.....	
3a NZL1	S.....GS	E.....	..IAL.....	.....	.....I.S..RG	M.L.....	.....T.....	.....C.....	.....F.....	.....	
3a HPCHK6	S.....GS	E.....	..IAC.....	.....	.....KM.S..RG	M.L.....	.....T.....	.....C.....	.....F.....	.....	
DX506	.....GS	E.....	..IAL.....	.....	.....I.S..RG	M.L.....	.....T.....	.....C.....	.....F.....	.....Y.....	
3b Tr	.....G.TS	D.....	L..AM.....	..V....E.	.....S..RG	M.V...F..	.....V.....	.....C.....	.....	.....T....	
10a JK049	S.....TG	E.....R.	..G.....	.....	.....KQ.TS	..V...F..	.....Q.....	.....C.....	.....I.....	.....	
4a ED43	S.....P.	.....	..L.....	.....	.....RQ.TS	..L.....	.....	.....C.....	.....F.....	.....	
DX499	.....P.	.....	..L.....	.....	.....RQ.TS	..L.....	.....	.....C.....	.....F...Y.	.....Y.....	
11a JK046	...Q.T..PL	...V.....	...Y.....	.....	.....Q.RT	..L...F..	..V.....	.....C.....	.....	.....	

C		2234	2244	2254	2264	2274	2284	2294	2304	2314
1a HCV-PT	GGNITRVESE	NKVVILDSFD	PLVAEEDE-R	EISVPAEILR	KSR-RFAQAL	PVWARPDYNP	PLVETWKKPD	YEPPVHVHGP	LPPPK	
1a HC-J1	.....	.....	.....	.....T...	..I.....	..I.....N.....	.....Q.....	.....	.....	
1b HCV-J	.....	.....	..IR.V.....	.....	..P.K.PP..	..I.....	..L.S..D..	..V.....	..ST.	
1b BK	.....	..V.....	..R.....	..V.....	..K.K.PA.M	..I.....	..L.S..D..	..V.....	..I.	
HCVUPCP	.....	..V.....	..R.....	..V.....	..K.K.PA.M	..I.....	..L.S..D..	..V.....	..I.	
1c HC-J9	.....	..I.....	.....D.....	.....L..	..K.K.PP.M	..I.....	..P..R..	..L.....	.....	
2a HC-J6	..DV..I..	S..V...L.	..M.E.RSD.L	..P.I.S.YML	PKK...PP..	..A.....	..S..R..	..Q.AT.A.A	.....	
2b HC-J8	..DV..I..	S..IV...L.	SMTEV..D..	..P..S.Y.I	..R.K.PP..	..P.....	..V.I...R.G	..Q.T.L.A.A	..TP	
2c BEBE1	..M..I..	S..LMV...V	..DK.....	..P.I.S.Y.L	PKS...PP..	..P.....	..L...R..	..Q...A.A	..G	
3a NZL1	..S.....	T...V...E	..R..T.D.V	..P..A..CFK	..PP.KYPP..	..I.....	..LDR..A..	..V..T...A	..RG	
3a HPCHK6	..S.....	T...V...E	..R..T.D.A	..L.AA..CFK	..PP.KYPP..	..I.....	..LDR..S..	..V..T...A	..KG	
DX507	..S.....	T...V...E	..R..T.D.A	..L..A..C.K	..PP.KYPP..	..I.....	..LDR..A..	..V..T...A	..QA	
3b Tr	..S.....	T...V...E	..R...D.T	..L.I...CFK	..PP.KYPP..	..I.....	..LPS..D.T	..A...A	..TR	
10a JK049	..S.....	S...E	..R.CD..D	..L..A..CFK	..PP.KYPP..	..I.....	..P..D..	..V..T...A	..Q.	
4a ED43	..STA....	TD E..I...E	SC...QNDD	..V..A....	PTK.K.PP..	..I.....	..T...QQ.	..QA.T...A	..A.	
11a JK046	.....	..I.....E	..K..F.D..	...A..CH.	PP.FKYPP..	.....	..L...A..	..D...S..A	..QG	

FIG. 1—Continued.

and 4a differed from each other by 14 amino acids. In the NS5 sequence there were 23 differences between genotypes 1b and 3a over a length of 85 amino acids (27% divergence). Six of these substitutions affected the charge of the protein: four substitutions of nonpolar amino acids for basic groups and two substitutions of acidic groups for nonpolar groups.

**Quantitation of antibody reactivity to core, NS3, and NS5 antigens.** The serological reactivities of sera from 33 individuals with genotype 1b, 34 individuals with genotype 3a, and 43 individuals with genotype 4a HCV infections to each of the recombinant antigens from genotypes 1b, 3a, and 4a were measured. To investigate the reproducibility of the method used to quantify antibody levels, each of the serum specimens was assayed in replicate against the type 1b and 4a core proteins (Fig. 2). A close correlation was observed between the two measured antibody levels, with nonparametric correlation coefficients being 0.877, 0.889, and 0.862 for type 1b, 3a, and 4a antisera, respectively. For the type 4a core antigens, the cor-

responding correlation coefficients were 0.784, 0.721, and 0.802.

There was also a close correlation between reactivity to the core protein of type 1b and that to type 4a (Table 2), with correlation coefficients of 0.834, 0.765, and 0.767 for type 1b, 3a, and 4a antisera, respectively. Significant correlations were also consistently observed between the reactivities of antisera to NS3 proteins of types 1b, 3a, and 4a, as well as between the reactivities of antisera to NS5 proteins of type 1b and 3a. In contrast, there was little if any correlation between antibody reactivity to different regions of the genome. For example, the reactivity of sera from type 1b-infected individuals to the type 1b core protein showed no correlation with reactivity to type 1b NS3 or NS5 proteins (correlation coefficients, -0.118 and -0.181, respectively; Table 2). The only exceptions were weak correlations (0.443 and 0.471) between the reactivities to 3a NS3 and NS5 (but only for type 1b sera) and between NS5 of type 1b with NS3 of 4a (restricted to type 3a sera).



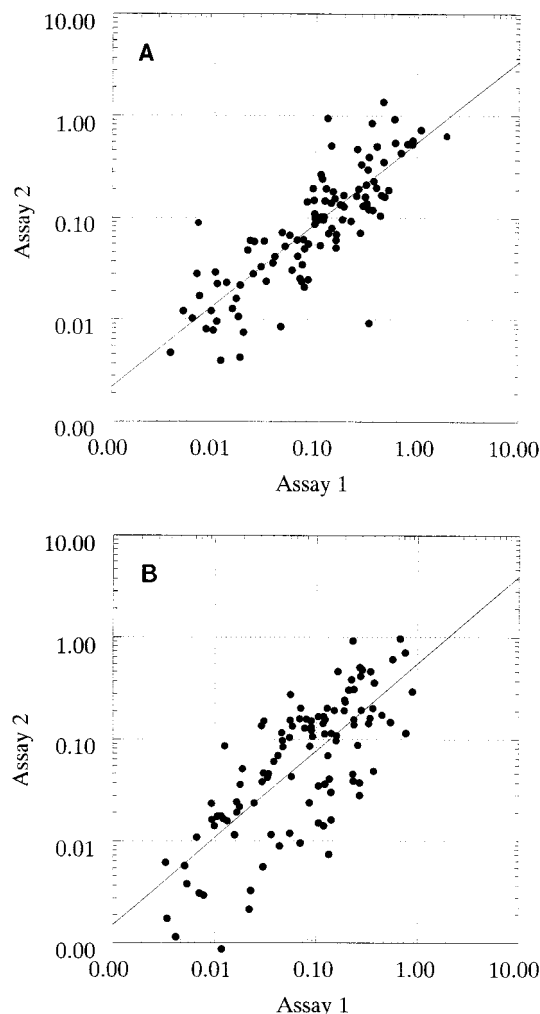


FIG. 2. Measurement of the reproducibility of measurement of levels of antibody to type 1b (A) and type 4a (B) core protein by sera from study subjects by repeat testing. The correlation is indicated by a regression line.

**Genotype dependence of serological reactivity.** Sera collected from individuals infected with different genotypes varied in their frequency of reactivity to NS3 and NS5 antigens of different genotypes (Table 3). For NS3, frequencies of reactivity for type 1b, 3a, and 4a antisera to type-homologous NS3 proteins were 85, 100, and 76%, respectively, compared with a range of 44 to 76% for type-heterologous combinations. Similarly, the frequency of reactivity of type 1b sera with type 1b NS5 antigen (65%) was higher than that of heterologous sera (47 and 51%), as was reactivity to type 3a NS5 (58% type-homologous reactivity, compared with 24 and 45% for type-heterologous combinations). In the core region, high frequencies of reactivity were observed for both type-homologous (95 to 97%) and type-heterologous (86 to 97%) combinations, indicating the greater antigenicity of this region of the genome and/or a greater proportion of shared epitopes between genotypes.

To compare the strength of reactivity with type-homologous and type-heterologous antigens, antibody levels relative to those for the positive control calculated from OD readings at dilutions ranging from 1:10 to 1:640 were used to calculate antibody levels relative to those for the positive control (Fig. 3). There were significant differences in antibody reactivity to

the type 1b core protein between samples from individuals infected with genotype 1b and those from individuals infected with genotype 4a ( $P = 0.033$ ) but not those with genotype 3a ( $P = 0.811$ ). The distribution of antibody levels directed to the genotype 4a core antigen showed no significant difference between genotypes.

Antibody reactivity against NS3 was frequently undetectable among sera from individuals infected with HCV with type-heterologous antigen (Table 3; Fig. 3C). The distribution of levels of antibody to type-homologous antigens was consistently greater than those to type-heterologous antigens. For example, the median level of antibody to type 1b NS3 in type 1b sera was 0.225, which was substantially greater than the median reactivities of type 3a and 4a sera to this protein (0.003 [ $P = 0.004$ ] and 0.02 [ $P = 0.005$ ], respectively). Similar, predominant type-specific reactivities against type 3a and 4a antigens were observed (Fig. 3D and E).

Only 54% of the samples reacted with the NS5 genotype 1b antigen and 43% reacted with the genotype 3a antigen. The median antibody level of genotype 1b sera (0.031) was eightfold higher than that for type 3a samples (0.004) and threefold higher than that for type 4a (0.012). The distribution of antibody levels among genotype 1b samples was significantly higher than that among genotype 4a samples ( $P = 0.029$ ) but not genotype 3a samples. The reactivity of type 3a sera against

TABLE 2. Correlation between reactivity to different antigens

Antigen and genotype	HCV genotype in serum	Correlation for the following antigens and genotypes <sup>a</sup> :					
		Core		NS3			NS5
		1b	4a	1b	3a	4a	1b 3a
Core 1b	1b	(1)					
	3a	(1)					
	4a	(1)					
4a	1b	<u>0.834</u>	(1)				
	3a	<u>0.765</u>	(1)				
	4a	<u>0.767</u>	(1)				
NS3 1b	1b	-0.118	0.023	(1)			
	3a	0.133	0.279	(1)			
	4a	0.100	-0.035	(1)			
3a	1b	-0.270	-0.066	<u>0.456</u>	(1)		
	3a	0.153	0.274	<u>0.363</u>	(1)		
	4a	0.159	0.030	<u>0.627</u>	(1)		
4a	1b	0.048	0.224	<u>0.710</u>	<u>0.518</u>	(1)	
	3a	0.215	0.130	<u>0.570</u>	<u>0.393</u>	(1)	
	4a	-0.058	-0.087	<u>0.497</u>	0.272	(1)	
NS5 1b	1b	-0.181	-0.220	0.150	0.275	0.157	(1)
	3a	0.066	0.000	0.218	0.265	<u>0.471</u>	(1)
	4a	0.058	-0.192	0.069	0.168	0.055	(1)
3a	1b	-0.141	-0.43	0.247	<u>0.443</u>	0.348	<u>0.630</u> (1)
	3a	0.051	0.172	0.216	0.266	0.202	<u>0.649</u> (1)
	4a	-0.076	-0.108	0.186	0.179	0.041	0.303 (1)

<sup>a</sup> Correlation is indicated by Spearman's rank correlation coefficient. Separate values are provided for antisera of each genotype. Significant values ( $P < 0.05$ ) are underlined.

TABLE 3. Frequency of reactivity to core, NS3, and NS5 proteins

Antiserum	No. of specimens reactive to the following antigen/total no. of specimens (%):					
	Core		NS3			NS5
	1b	4a	1b	3a	4a	1b 3a
Type 1b	32/33 (97)	32/33 (97)	28/33 (85)	25/33 (76)	24/32 (76)	21/33 (64) 8/33 (24)
Type 3a	33/34 (97)	30/34 (88)	15/34 (44)	33/33 (100)	9/33 (27)	16/34 (47) 18/31 (58)
Type 4a	37/43 (86)	41/43 (95)	31/43 (72)	27/43 (63)	31/41 (76)	22/43 (51) 19/42 (45)

NS3 of genotype 3a was significantly greater than that of the type 1b or 4a sera ( $P = 0.002$  and  $0.01$ , respectively).

**Ratio of type-specific and cross-reactive reactivities.** The ability to measure the reactivity of the same antiserum to

antigens of different genotypes allowed for an estimation of the relative levels of genotype-specific and cross-reactive serological reactivities. For the core proteins, the levels of antibody to the genotype 1b antigen in each of the type 1b and 4a sera were

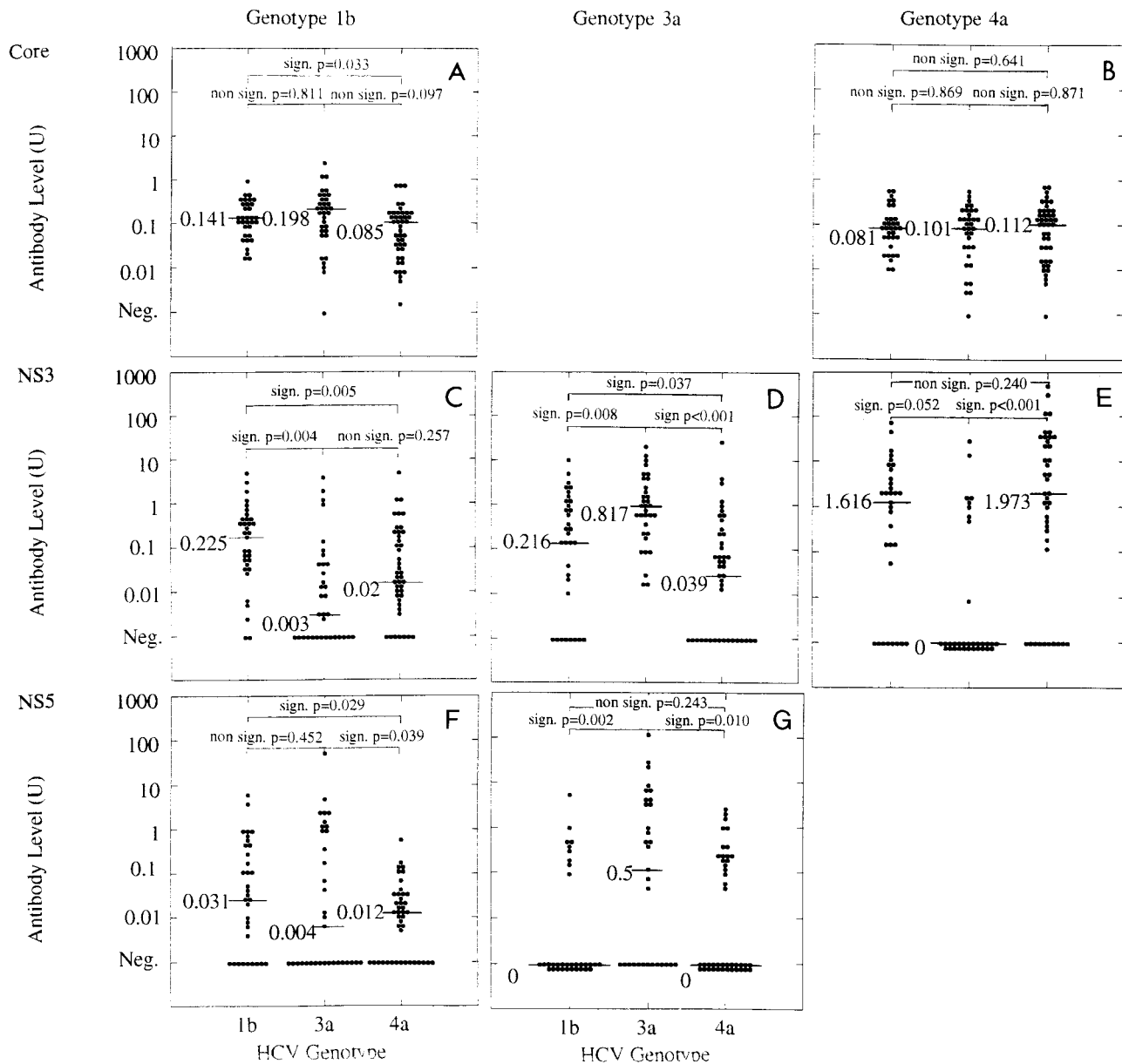


FIG. 3. Distribution of antibody reactivity of sera from individuals infected with type 1b, 3a, and 4a to core proteins of genotypes 1b and 4a (A and B, respectively); NS3 of genotypes 1b, 3a, and 4a (C, D, and E respectively); and NS5 of types 1b and 3a (F and G, respectively). Median values are indicated by short horizontal bars. Pairwise comparison of the distributions of values was carried out by the nonparametric Kruskal-Wallis test.

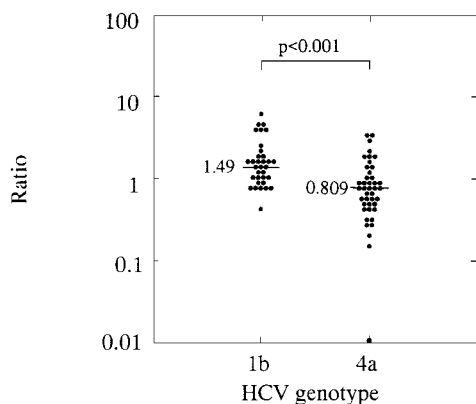


FIG. 4. Ratios of reactivity of type 1b and 4a sera to type 1b/type 4a core antigens. Median ratios are indicated by short horizontal bars. Pairwise comparison of the distributions of values was carried out by the Kruskal-Wallis test. Ratios of reactivities to other antigens are presented in Table 4.

divided by their levels to the genotype 4a antigen (Fig. 4; Table 4). The median ratio of reactivity to type 1b/reactivity to type 4a antigens for type 1b antisera was 1.49, compared with a median ratio of 0.809 for the type 4a antisera. This indicates that type 1b sera react more strongly against the type 1b (homologous) core protein, while the type 4a sera react slightly more strongly against the type 4a antigen. If it were shown that both core proteins were coated equivalently on the solid phase and showed equal antigenicity, then the type-specific reactivity to these proteins could be calculated from the difference from the ratio of 1 that would be expected from exclusively type-common reactivity. However, it is possible that some antigens are present at higher available concentrations than others through differences in binding to the solid phase or solubility. These differences were taken into account by using the derivation described in Materials and Methods. In this instance, the term  $E_{AB}/E_{AA}$  represents the median of the ratio of reactivity between type 4a antisera with type 1b and type 4a antigens (0.809). The term  $E_{BB}/E_{BA}$  is similarly represented by the median ratio of the type 1b antisera (1.49). Therefore, the proportion of type-common reactivity can be calculated as 0.74, with type-specific reactivity forming the remainder of the reactivity (0.26).

The median ratio of reactivity of the type 3a sera to type 3a and 1b NS5 antigens (0.654) was substantially lower than that of type 1b sera (median, 4; Table 4). Therefore, the proportions of type-common and type-specific reactivity were 40 and 60%, respectively; i.e., the majority of the serological reactivity to this antigen was type specific. Finally, three sets of pairwise comparisons can be made for the NS3 region, in which antigens for all three genotypes were available. Sixty-two percent of the serological reactivity between type 1b and 3a proteins was type specific, similar to the proportions of 61% between type 1b and 4a proteins and 77% between type 3a and 4a proteins (Table 4).

## DISCUSSION

**Antigenic variability of HCV.** The aim of this study was to investigate the degree of type-specific serological reactivity to antigens used in current, third-generation screening assays. The use of recombinant antigens expressed from different genotypes in the enzyme immunoassay allowed reciprocal measurements of type-homologous and type-heterologous reactivity to be made, and these provided a more rigorous assessment

of the type-specific components of reactivity to the core, NS3, and NS5 regions. This addresses a potential criticism of previous investigations that showed weaker reactivity of sera from individuals infected with non-type 1 genotypes in either of the screening ELISAs or to individual antigens in the confirmatory recombinant immunoblot assay (5, 8, 12, 14, 21, 28), in that there is a possibility that these observations resulted from a generally weaker serological response to infection than that elicited by type 1. In the current study we were able to consistently show stronger reactivity of sera to antigens of a homologous type than to antigens of heterologous types.

From pairwise reciprocal measurements of antibody reactivity (such as type 1b and 4a antisera against type 1b and 4a antigen), it was possible to quantify the relative contributions of type-specific and cross-reactive antibody reactivities by using the relation derived in Materials and Methods (Table 4). These calculations were independent of possible differences in the strength of the serological response elicited by infection with different genotypes and were also independent of differences in antigen concentration or overall antigenicity between proteins of different genotypes (expressed as the ratio  $A_{AB}$ ). This removes the potential criticism that the coating efficiency of antigens from different genotypes onto the solid phase was not compared prior to measurement of antibody levels. From this analysis we found a relationship between the degree of amino acid sequence divergence between recombinant proteins and their degree of cross-reactivity. The sequence of the core protein was the most conserved and the core protein showed approximately 25% type-specific reactivity, while the more divergent sequences, those of the NS3 and NS5 regions, showed substantially greater proportions of type-specific reactivity. These results are consistent with previous comparisons of the type-specific component of reactivity to peptides corresponding to linear epitopes in NS4. By absorption in solution with peptides of heterologous genotypes, it was shown that reactivity to type-homologous peptides was reduced but was rarely eliminated (1, 26), allowing development of a sensitive and specific serological typing assay. The type-specific component of serological reactivity to NS4 and, in some studies, to the core protein has been also used in typing assays without cross-absorption, because it has consistently been observed that reactivity to type-homologous antigens in these (13, 19, 22) and other regions (29) is stronger.

**Implications for screening assays.** The effect of the demonstrated antigenic variabilities of the components of third-gen-

TABLE 4. Type-specific and type-common serological reactivity to HCV core, NS3, and NS5 antigens

Region	Anti-serum		Median ratio <sup>a</sup>		<i>P</i> value <sup>b</sup>	Percent		
	A	B	A	B		<i>T<sub>c</sub></i>	<i>T<sub>s</sub></i>	Amino acid divergence <sup>c</sup>
Core	4a	1b	0.809	1.49	<0.001	74	26	6.4
NS5	3a	1b	0.654	4.0	0.014	40	60	27
NS3	3a	1b	0.053	0.373	0.001	38	62	16
	4a	1b	0.016	0.107	<0.001	39	61	14
	4a	3a	0.049	0.939	<0.001	23	77	15

<sup>a</sup> Ratio of reactivity to antigens of genotypes A and B.

<sup>b</sup> Comparison of the distribution of ratios of reactivity of individual antisera to antigens of genotypes A and B by the Kruskal-Wallis nonparametric test.

<sup>c</sup> Degree of amino acid sequence divergence between antigens of genotypes A and B.



eration screening assays on their overall sensitivity for screening is difficult to estimate with precision for two reasons. Individuals vary in their serological responses to different antigens, and therefore in the extent to which these responses may result in cross-reactivity with antigens of heterologous genotypes. For example, if reactivity were directed solely to the core protein, this protein would cross-react with antigens of heterologous genotypes, whereas sera monoreactive with NS3, such as is found upon seroconversion, would be expected to be predominantly type specific.

Furthermore, each of the three antigens investigated is likely to contain a range of linear and conformational epitopes, and these will vary in their degree of cross-reactivity. Recognition of different epitopes in the antigens may be one explanation for the wide range of ratios of reactivity to type-homologous and type-heterologous antigens observed between sera of the same genotype (Fig. 4). In the extreme case, it is possible that reactivity confined to epitopes in the core protein that are type specific would lead to poor or absent reactivity to the core protein of other genotypes. Conversely, the high degree of cross-reactivity observed between certain sera with NS3 or NS5 proteins of heterologous genotypes may have resulted from their recognition of shared epitopes.

The observed differences in reactivity of sera to homologous and heterologous proteins would only lead to false-negative results on serological screening if antibody levels in samples to be tested were close to the cutoff sensitivity of the assay. In a previous study, we measured antibody reactivity in the Ortho third-generation assay and found a wide range of antibody levels (approximately 5,000-fold) among samples from blood donors infected with genotypes 1, 2, and 3, with the lowest level found to be 0.0007, which is just above the cutoff value of the test (12). Low antibody levels and reactivity to a restricted range of epitopes, such as those in NS3, are found in acutely infected individuals (18), and it is likely that earlier detection of seroconversions, and therefore a reduction in the window period associated with non-type 1 infection, may be achieved by assays containing NS3 and other antigens from a wider range of genotypes.

The finding of significant antigenic variability of antigens used for serological screening will form the basis for a number of future investigations. Now that antigens from other genotypes have been produced, it will be possible to carry out large-scale screening of populations infected with non-type 1 genotypes (for example, with the type 4a antigens for testing individuals in the Middle East). This may reveal the frequency with which anti-HCV samples are being missed by conventional assays and may ultimately help reduce the frequency of posttransfusion hepatitis further, particularly when populations with a high frequency of acute infection are screened. Similarly, the incorporation of antigens from other genotypes (particularly NS3) in confirmatory assays may resolve the results for a number of the indeterminate samples identified in blood donor screening and for which interpretation of results is currently problematic.

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